The small leucine-rich proteoglycan biglycan modulates BMP-4-induced osteoblast differentiation

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ABSTRACT **Biglycan (bgn) is a small leucine-rich proteoglycan enriched in extracellular matrices of skeletal tissues. *Bgn*-deficient mice develop age-related osteopenia with a phenotype that resembles osteoporosis and premature arthritis. In the present study, we have examined the differentiation of *bgn*-deficient osteoblasts from neonatal murine calvariae and found that the absence of bgn caused less BMP-4 binding, which reduced the sensitivity of osteoblasts to BMP-4 stimulation. The loss of sensitivity resulted in a reduction of Cbfa1 expression, which ultimately led to a defect in the differentiation of osteoblasts. However, the response of *bgn*-deficient osteoblasts to BMP-4 was completely rescued by reintroduction of biglycan by viral transfection. We propose that biglycan modulates BMP-4-induced signaling to control osteoblast differentiation.—Chen, X.-D., Fisher, L. W., Robey, P. G., Young, M. F. The small leucine-rich proteoglycan biglycan modulates BMP-4-induced osteoblast differentiation. *FASEB J.* 18, 948–958 (2004)**

Key Words: microenvironment · osteoblastic progenitors

OSTEOBLASTS ARISE from mesenchymal stem cells that pass through a series of maturational stages on their way to becoming mature functional cells in a manner similar to the differentiation of other cell types such as hematopoietic cells (1). However, bone is a unique organ that is composed of a massive amount of matrix and a relatively small number of cells compared with other organs. The microarchitecture of the extracellular matrix (ECM) in bone has been extensively studied, and the major role of the ECM is considered to be its contribution to biomechanical strength (2–6). In contrast, little is known about the role of the ECM on osteogenic cells during the process of maturation. There are several reports that type I and II collagens and the small leucine-rich proteoglycans, including biglycan (bgn) and decorin (dcn), can bind growth factors (7–9). Based on this evidence, a theory has arisen that the ECM may play an important role as a reservoir for balancing the activities of growth factors to control osteoblast differentiation.

To better understand how proteoglycans may modulate growth factor activities and subsequent stem cell maturation, we used biglycan-deficient mice (*bgn*-KO) as an animal model for our investigation. Bgn is a member of the family of proteoglycans known as small leucine-rich proteoglycans (SLRPs) (10, 11). It consists of a 45 kDa core protein made of leucine-rich repeats that are ~25 amino acids long (12). Bgn has two glycosaminoglycan (GAG) chains attached to its amino terminus and two cysteine clusters flanking each end of the leucine repeats that are engaged in disulfide bond formation. The bgn gene is located on the X chromosome (13). Bgn protein is highly expressed in the extracellular matrices of bone and cartilage and is localized at the cell surface (14). It may interact with growth factors, including TGF-β, to regulate their biological activity (7). Mice with targeted disruption of the *bgn* gene developed age-dependent osteopenia (15). Further in vitro analysis showed that bone marrow stromal cells isolated from *bgn*-KO mice had multiple defects including increased apoptosis, reduced numbers of osteoblast progenitors (CFU-OB), as well as decreased collagen mRNA and protein. A possible explanation for these abnormalities may be that *bgn*-KO bone marrow stromal cells respond poorly to TGF-β (16).

Because BMPs also play an important role in osteoblast differentiation, our hypothesis in the present study was that bgn might regulate BMP-stimulated osteoblastic differentiation. To test this hypothesis, we examined the response of *bgn*-KO osteoblastic cells to BMP-4. Since there are no specific surface markers to isolate osteoblastic stem cells from bone marrow, we selected the well-described in vitro culture system of neonatal murine calvarial cells, which provide a reproducible population of preosteoblasts (17). Our data strongly suggest that bgn modulates BMP-4 binding. It was shown that bgn deficiency affected BMP-4 signal transduction, causing a reduction in Cbfa1, which in turn led to defective osteoblast differentiation. This is the first report to demonstrate that bgn modulates BMP-stimulated osteoblastic differentiation. We propose that bgn is required for retaining an appropriate microenvironment for the maturation of osteoblastic stem cells.

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MATERIALS AND METHODS

Animals

All experiments were performed using 2- to 5-day-old *WT* and *bgn*-KO mice (C3H) according to institutionally approved guidelines (IRB #001-151). The genotype of the *WT* and *bgn*-KO mice was determined by PCR as described previously (16).

Culture medium

The complete medium consisted of α-modified minimum essential medium (Life Technologies, Grand Island, NY, USA), glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL, Biofluids, Rockville, MD, USA), 2-mercaptoethanol (10^{-4} M), and 10% fetal bovine serum (FBS, Becton Dickinson, Franklin Lakes, NJ, USA). The differentiation medium was the complete medium supplemented with 2 mM β-glycerophosphate (Sigma Chemical Company, St. Louis, MO, USA) and 10^{-4} M L-ascorbic acid phosphate magnesium (Wako Chemicals, Richmond, VA, USA).

Reagents

Human recombinant BMP-4 (hrBMP-4) and biotinylated anti-BMP-2/4 antibody were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The antibody against Cbfa1 (AML3) was purchased from Oncogene Research Products (Cambridge, MA, USA). The antibody against phosphorylated SMAD1 (p-SMAD1) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against BMP-4 receptor type I (BMPR-IA and BMPR-IB) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of murine calvarial cells

Neonatal murine calvarial cells were prepared as described previously (18). Briefly, calvariae harvested from 3- to 5-day-old *WT* or *bgn*-KO mice were pretreated with 4 mM EDTA in PBS for 2 × 10 min. The calvariae were digested using CLS-2 bacterial collagenase (Worthington Biomedical Corporation, Freehold, NJ, USA) at 200 U/mL in PBS for 5 × 10 min. Cells from the last three digestions were collected and served as the starting population of cells that are highly enriched in osteoblastic cells (17). The cells were washed twice in complete culture medium, seeded into 100 mm dishes at a density of 1000–2000 cells/cm^2, and cultured at 37°C in the presence or absence of BMP-4 (30 ng/mL). Once confluence was reached (~day 7), cultures were fed twice weekly with the differentiation medium. The cells were collected from cultures at various intervals as indicated in the following experiments.

RNA extraction and Northern blot analysis

Mouse cDNA probes, including biglycan, decorin, type I collagen, osteopontin, and bone sialoprotein (BSP), were generated at the NIDCR (http://csdb.nidcr.nih.gov); osteocalcin was a gift from Dr. Caren Gundberg (Yale University, New Haven, CT, USA). The probes were radiolabeled to specific activities of ~1×10^9cpm/μg using ^{32}P-dCTP and a random primer DNA labeling kit (Stratagene, Cedar Creek, TX, USA).

The cells were collected from culture on days 4, 7, 14, and 28 after initial seeding. Total RNA was isolated using RNA STAT-60 Reagent (TEL-TEST "B". Inc., Friendswood, TX, USA) according to the manufacturer's protocol.

RNA (10 μg) was denatured in 1.8% of formamide/formaldehyde, electrophoresed on 1.2% agarose gels in 1 × MOPS running buffer (ONCOR, Gaithersburg, MD, USA), transferred to Zeta-Probe™ GT Genomic Tested Blotting Membrane (Bio-Rad, Hercules, CA, USA), cross-linked to the membrane by exposure to 1200 μjoules of short wave UV irradiation using a UV Stratalinker 1800 (Stratagene), prehybridized for 3 h at 37°C in 15 mL of Hybrisol I hybridization buffer (ONCOR), then hybridized for 20 h with 1–2 × 10^6 cpm of labeled probe per milliliter of hybridization buffer. After hybridization, filters were washed and exposed overnight at –70°C. Equal loading RNA was determined by probing blots with cDNA to β-actin.

Real-time RT-PCR

One microgram total RNA from the sample preparation was reverse transcribed with 50 units of SuperScript II RT using random hexamer primers (Nitrogen Life Technology) according to the manufacturer's instructions.

The primers used to amplify were designed with Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). They were osteocalcin: F5′-cctcttgaaagagtgggctg-3′, R5′-cctcgggagacaaacaacat-3′, giving a product size 277 bp and T_m 59.98°C; bone sialoprotein (BSP): F5′-attttgctcagcattttggg-3′, R5′-ctgaagagtcactgcctccc-3′, giving a product size 196 bp and T_m 60.07°C.

The LightCycler PCR reaction mix was prepared according to manufacturer's instruction (Roche Diagnostics, Indianapolis, IN, USA). Master mix (18 μL) was placed into the glass capillaries and 2 μL of template DNA was added. Capillaries were placed into the rotor of the LightCycler (Roche Diagnostics Corp.). After a denaturation step at 95°C for 10 min, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 10 s, hybridization at 59°C for 5 s, and elongation at 72°C for 10 s. A total of 45 cycles were performed.

The amount of mRNA was calculated for each sample from the standard curve using the instrument software. Results were expressed as the ratio of the amount of tested mRNA divided by GAPDH mRNA (19).

Calcium accumulation

Neonatal murine calvarial cells from *WT* or *bgn*-KO animals were plated into 6-well plates at a density of 1000–2000 cells/cm^2 in 2 mL of the complete medium and grown to confluence. Then the cells were continuously cultured in the presence of differentiation medium in the presence or absence of BMP-4 (30 ng/mL). The media was replaced twice a week. After 4 wk postconfluence, the cells were stained with Alizarin Red to visualize calcium deposition using a described method (20). To quantify the calcium content, Alizarin Red bound to the cultures was extracted and measured spectrophotometrically as described previously (21).

Western blot analysis

Confluent cells were cultured in medium with 2% serum overnight and treated with BMP-4 (30 ng/mL) for various intervals as specified in the text. Then the cells were washed with PBS and lysed in an extraction buffer (25 mM Tris-Cl, pH 7.2, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 0.1 M NaCl, 1 mM EDTA) containing a protease inhibitor cocktail (Cat #: 1697498, Roche). Equal amounts of cell extracts (30 μg) were resolved on 10% NuPAGE Bis-Tris Gel

(Invitrogen Corp., Carlsbad, CA, USA), transferred onto nitrocellulose membranes (Schleicher and Schuell Inc., Keene NH, USA), and probed with antibodies as recommended by the manufacturer. An antibody against the mouse bgn (LF-105) was used at a 1/200 dilution to confirm the success of bgn rescued (22).

Luciferase activity assay

Cells were seeded into 24-well plates at 2×10^4 cells/well in complete medium, cultured until 70% confluence, and transfected with 6 μg/well of p6OSE2-Luc, a gift from Dr. Gerard Karsenty (Baylor College of Medicine, Houston, TX, USA) (23), and 20 ng/well of the control plasmid pRL-SV40 using the Calcium Phosphate Transfection Kit (Promega Corp., Madison, WI, USA) according to the manufacturer's recommendations; three wells were set up for each condition. After 16 h, the medium was replaced with fresh complete medium. After a 24 h recovery, the cells were treated with vehicle or BMP-4 (30 ng/mL) in medium containing 2% FCS for 3 h. The cells were harvested; luciferase activity in cell lysates was determined with a luciferase assay system (Promega Corp., Madison, WI, USA) using a Monolight 2010 for detection (Analytical Luminescence Laboratory, San Diego, CA, USA).

Infection with *bgn*-adenovirus (adv/*bgn*)

An adenovirus expressing bgn (clone 149E) was constructed using the full coding region of the human bgn cDNA (P16 in http://csdb.nidcr.nih.gov/csdb/frame_reagents.htm). cDNA was cloned into the shuttle vector pACEF1.pLpA (24) and orientation/integrity determined by DNA sequencing. Adenoviruses were plaque purified and screened for protein expression by Western blotting using antibody LF 51 (see above website). The virus was amplified in HEK 293 cells and purified through CsCl centrifugation using standard procedures. An adenovirus without a transgene was propagated in a similar manner and used as a negative control. Viral titer was estimated by optical density at Å260 and by plaque assay as described previously (24). Several different MOI (multiplicity of infection) were tested to determine optimal expression in the calvarial cell system. Comparable MOI of wild-type ("empty vector") virus were used as negative controls.

To rescue *bgn*-KO cells, the cells were seeded into 24-well plates at 2×10^4 cells/well in complete medium, cultured until 30% confluence, and infected with either 3.75×10^7 PFU/mL of Adv/bgn or the same concentration of recombinant adenovirus without the *bgn* gene/cDNA (adv/Emp) for 72 h. The infected cells were tested as specified in the text.

Measurement of ALP activity

Confluent cells were incubated for 48 h in the presence or absence (control) of 30 ng/mL BMP-4 in serum-free medium and lysed in lysis buffer (20 mM Tris, 0.5 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 0.1% Triton X). ALP levels in the lysates were determined using an alkaline phosphatase kit (Sigma Chemical Co.) and production of ρ-nitrophenol was measured by spectrophotometric absorbency at 405 nm. The ALP value was calculated using standards and expressed as Sigma units per milligram of protein lysates. One Sigma unit is equal to 1 μM ρ-nitrophenol per hour.

Immunohistochemistry

The cells were seeded into 2-well chamber slides at 2×10^4 cells/well in complete medium and cultured until confluence (7 days). The cells were fixed for 30 min in 4% phosphate-buffered formaldehyde at room temperature (RT), and washed and preblocked in PBS with 0.1% BSA, 5% normal goat serum (blocking solution) for 30 min at RT. To detect BMP-4 binding, the cells were preincubated with BMP-4 (10 μg/mL) for 2 h at RT and washed with PBS three times. Cells not treated with BMP were used to distinguish endogenous BMP and served as negative controls. The BMP-4-positive cells were identified by incubation of biotinylated anti-BMP-2/4 antibody (2.5 μg/mL) for 1 h and subsequently streptavidin-FITC (5 μg/mL) for 30 min at RT. To detect endogenous mouse bgn or dcn by single-color immunofluorescence staining, cells were incubated with primary antibody, 1:300 dilution of rabbit anti-mouse bgn antibody (LF-159) or rabbit anti-mouse dcn (LF-113) for 1 h (22), then washed and incubated with Rhodamine Red or FITC-conjugated goat anti-rabbit antibody (Accurate Chemical and Scientific Corp., Westbury, NY, USA), respectively, for 30 min at RT. For 2-color immunofluorescence staining, the cells pretreated with BMP-4 were incubated with biotinylated anti-BMP-2/4 antibody for 1 h and subsequently incubated with streptavidin-FITC for 30 min at RT. After washing, the cells were incubated with rabbit anti-bgn antibody (LF-159) for 1 h at RT, washed, then incubated with Rhodamine Red-conjugated goat anti-rabbit antibody for 30 min at RT. After staining, the cells were washed and mounted in Mounting Medium with DAPI to stain the nuclei (Vector Laboratories, Inc., Burlingame, CA, USA).

Flow cytometry

Single-cell suspensions were prepared from monolayer cultures by trypsin treatment (0.05% for 2 min at 37°C), followed by two washes in cold PBS with 2% FCS. The cells were preincubated with BMP-4 (10 μg/mL,) for 2 h at 4°C. After washing with PBS three times, cells ($5–10 \times 10^5$) were incubated in 100 μL of biotinylated anti-BMP-2/4 antibody (2.5 μg/mL) for 30 min. at 4°C. The stained cells were washed twice in staining buffer (PBS with 5% FCS and 0.01% sodium azide) and incubated in 50 μL of streptavidin-FITC (5 μg/mL) for 20 min at 4°C, then washed twice with staining buffer and immediately analyzed by flow cytometry or fixed with 1% paraformaldehyde in PBS and analyzed within 96 h. Cells stained with antibody to BMP-4 without preincubation with BMP served as negative controls. Cell suspensions were analyzed using a Becton Dickinson FACSstarPlus flow cytometer.

Statistical analysis

The results were expressed as the mean ± standard deviation (M±SD), calculated from 3–6 independent experiments. Statistical analysis between experimental groups was performed by one-way ANOVA with InStat 2.01. Differences of $P < 0.05$ were considered significant. The data from Western blot analysis, immunohistochemical staining, and FACS analysis were reproduced in five separate experiments.

RESULTS

A defect in the differentiation of *bgn*-KO osteoblastic cells

We used the well-described in vitro culture system of calvarial osteoblastic cells (17) to compare the differentiation capacity of *bgn*-KO and wild-type (*WT*) cells. The expression of specific bone extracellular matrix

and proteins was determined during differentiation in culture of *WT* and *bgn*-KO neonatal murine calvarial cells on days 4, 7, 14, and 28 of culture. Northern blot analysis showed that osteopontin, BSP, and osteocalcin mRNA expression were sequentially decreased in the *bgn*-KO cells compared with *WT* (**Fig. 1*A***). BSP and osteocalcin mRNA were present in *WT* cells but barely detectable in the *bgn*-KO on day 28 of culture. While osteopontin mRNA had a different temporal pattern compared with BSP and osteocalcin appearing by day 7, it was always diminished in *bgn*-KO cells. Type I collagen, decorin, and fibromodulin all increased with time in culture in *WT* and *bgn*-KO, and there was no dramatic difference between *WT* and *bgn*-KO at any of these times (data not shown). To test the calcium accumulation capacity of the cells, we cultured them in the presence of BMP-4, a growth factor known to stimulate cells to progress toward the differentiation and mineralization stages of maturation (25). After 4 wk of culture, cells were stained with Alizarin Red (Fig. 1*B*). Only cells that were treated with BMP-4 showed significant levels of calcium accumulation, and *bgn*-KO cells accumulated less calcium than *WT* cells (Fig. 1*B*). When Alizarin Red was quantified using a spectrophotometer, we found that *bgn*-KO cells had ~30% less Ca^{2+} accumulation than *WT* cells (Fig. 1*C*).

Biglycan deficiency decreased the BMP-4 sensitivity

To determine how *bgn*-KO cells respond to BMP-4, the neonatal murine calvarial cells from *WT* and *bgn*-KO cells were cultured in the presence or absence of BMP-4 for 2 wk postconfluence. Real-time PCR was performed to determine the relative amount of BSP and osteocalcin mRNA expression. **Figure 2** shows that *bgn*-KO cells treated with BMP-4 did not significantly increase either BSP or osteocalcin expression compared with *WT* cells. BMP-4 induction of BSP and osteocalcin mRNA was completely rescued by infecting the *bgn*-deficient cells with adenovirus engineered to contain an expressing *bgn* gene (adv-*bgn*). Results suggested that the decreased calcium accumulation noted in *bgn*-KO cells treated with BMP-4 was due to their inability to upregulate BSP and osteocalcin appropriately.

Reduction of Cbfa1 expression in *bgn*-KO osteoblasts

Studies have indicated that the transcription factor Cbfa1 controls the expression of osteopontin, BSP, and osteocalcin (26–28). Therefore, we examined Cbfa1 levels in *bgn*-KO cells at protein and transcriptional levels by Western blot analysis and luciferase activity assay, respectively (**Fig. 3**). Based on our earlier studies using neonatal murine calvarial cells, we detected the 45 kDa form and never the larger forms at 7 days of culture. The 45 kDa species can be knocked down using Cbfa1-specific siRNA, providing proof of its identity. The observation is consistent with those reported by others using similar culture systems (29). In the present study, the 45 kDa Cbfa1 levels were diminished in

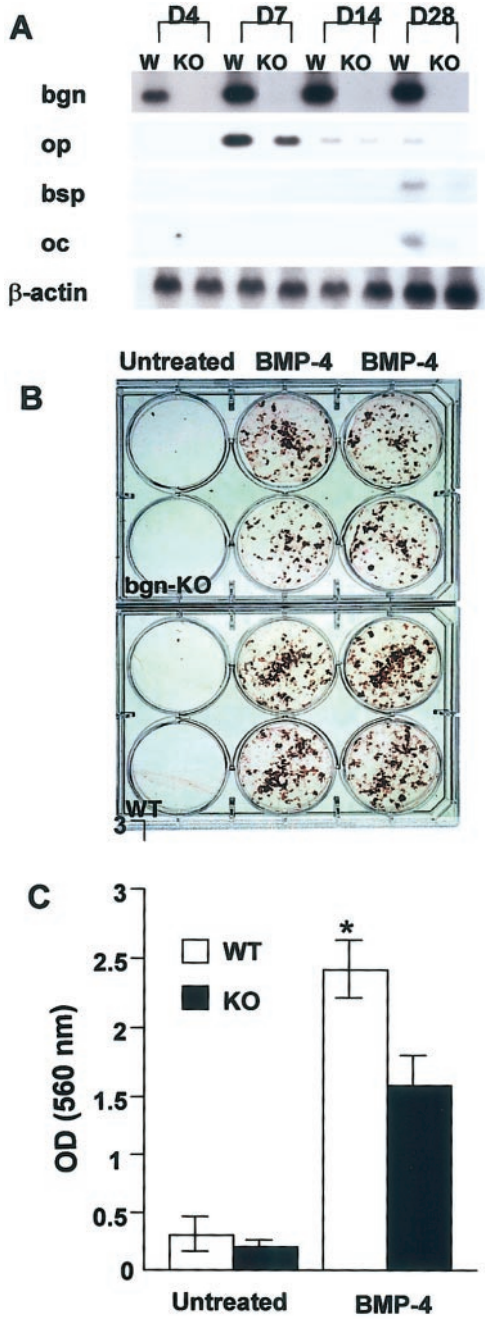

Figure 1. A defect in the differentiation of *bgn*-KO osteoblastic cells. *A)* Northern blot analysis of biglycan (bgn), osteopontin (op), bone sialoprotein (bsp), and osteocalcin (oc) mRNA expression by neonatal murine calvarial cells on days 4 (D4), 7 (D7), 14 (D14), and 28 (D28) of culture. The blot was stripped and reprobed with a radiolabeled cDNA for the gene β-actin (lower panel), demonstrating equal RNA loading. *B)* Ca^{2+} accumulation demonstrated by Alizarin Red staining (red color) in neonatal calvarial cell cultures from *WT* and *bgn*-KO mice. The cells were cultured for 4 wk postconfluence in the presence or absence of BMP-4. *C)* Quantity of extracted Alizarin Red by absorbency at 562 nm using a spectrophotometer. $*P < 0.05$ compared with accumulated calcium in *WT* and *Bgn*-KO (KO) cells in the presence of BMP-4.

bgn-KO cells with or without BMP-4 treatment compared with *WT* cells (Fig. 3*A*). To further determine the functional consequence of the decreased level of

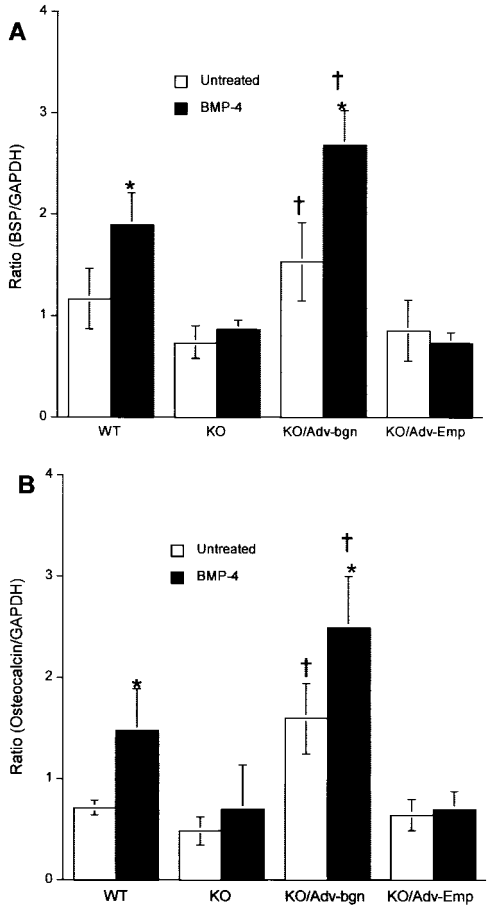

Figure 2. Bone sialoprotein (BSP) and osteocalcin were determined by relative quantification with real-time PCR in osteoblastic cells from wild-type (WT) and *bgn*-KO (KO) mice. Cells were cultured in the presence or absence of BMP-4 for 2 wk postconfluence. Results are expressed as the ratio of BSP/GAPDH (*A*) or osteocalcin/GAPDH (*B*). For the rescue, the *bgn*-KO cells were infected with *bgn*-adenovirus (adv/*bgn*) or wild-type virus (KO/Adv-Emp) as a negative control. $*P < 0.05$, $n = 4$ compared with the ratio between the untreated and BMP-4 treated (untreated WT vs. BMP-4 treated WT; untreated KO vs. BMP-4 treated KO). $\dagger P < 0.05$, $n = 4$ compared with *bgn*-KO cells between the rescued and unrescued with or without BMP-4 (KO vs. KO/Adv-*bgn* without the treatment of BMP-4, and KO vs. KO/Adv-*bgn* with the treatment by BMP-4).

Cbfa1, we transfected cells with the p6OSE2-Luc plasmid, which contains six copies of the consensus sequence (OSE2) for Cbfa1 linked to a luciferase reporter. Analysis of luciferase activity levels showed that BMP-4 treatment resulted in elevated activity in *WT* cells but not in *bgn*-KO cells (Fig. 3*B*).

Partial block of BMP-4 signal transduction in *bgn*-KO Cells

We next examined BMP-4 signal transduction in *bgn*-KO vs. *WT* cells by measuring phosphorylated SMAD1 (p-SMAD1). Once the cells reached confluence, they were treated with BMP-4 for 5, 10, 30 min and 2 h, and p-SMAD1 levels were determined by Western blot analysis. The level of p-SMAD1 reached maximal levels 30 min after BMP-4 treatment; at that time the p-SMAD1 was higher in *WT* than *bgn*-KO cells (**Fig. 4*A***, lane 3 vs. 4, respectively). To determine whether the decrease in p-SMAD1 in *bgn*-KO cells could be rescued by the addition of bgn, we infected the cells with Adv-*bgn*. We found that p-SMAD1 levels were rescued by the Adv-*bgn* infected KO cells to a level even higher than *WT* cells (Fig. 4*A*, lane 5). The success of infection and subsequent rescue of bgn expression was confirmed by Western blot analysis shown in Fig. 4*B*. A band corresponding to bgn was seen in the Adv-*bgn* infected bgn-KO cells (Fig. 4*B*, lane 5) compared with the positive controls from *WT* cells (Fig. 4*B*, lanes 1, 3). The expression of bgn by the Adv-*bgn* infected *bgn*-KO cells was observed mainly in the cytoplasm as judged by immunohistochemical staining (Fig. 4*C*). No staining was observed in bgn-KO cells infected with adenovirus

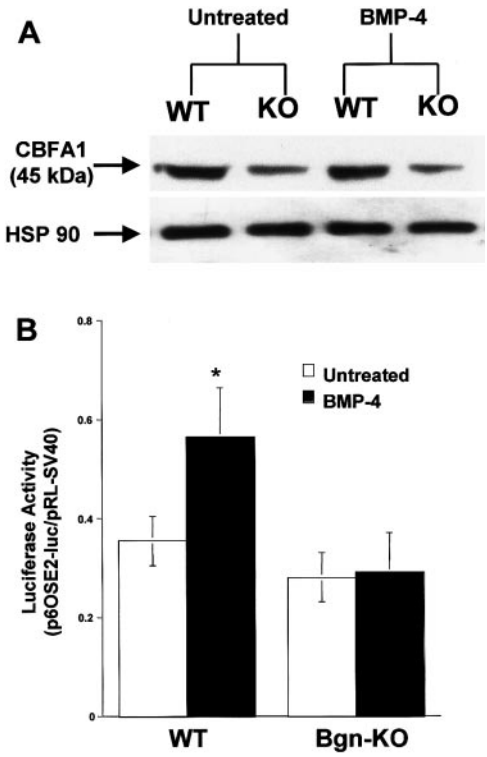

Figure 3. Reduction of Cbfa1 in *bgn*-KO cells compared with *WT* cells. *A)* Western blot analysis of Cbfa1 protein levels in *WT* and *bgn*-KO neonatal murine calvarial cell cultures. The cells were cultured until confluence and treated with BMP-4 for 24 h. A band of 45 kDa corresponding to Cbfa1 was detected. WT = wild-type; KO = *bgn*-KO. Equal protein loading is demonstrated by probing the same blot with a polyclonal antibody to HSP90 (bottom panel). *B)* Luciferase activity assay for detecting Cbfa1 transcriptional activation in *WT* and *bgn*-KO neonatal murine calvarial cell cultures. Cells were transfected with p6OSE2-luc for 16 h and pRL-SV40 as a control for transfection efficiency. Transfected cells were treated with BMP-4 for 3 h prior to luciferase analysis. Levels of transcriptional activation are represented as luciferase activity = ratio of p6OSE2-luc/pRL-SV40. $*P < 0.05$ compared with activity of cells in the presence of BMP-4 with that of cells in the absence of BMP-4.

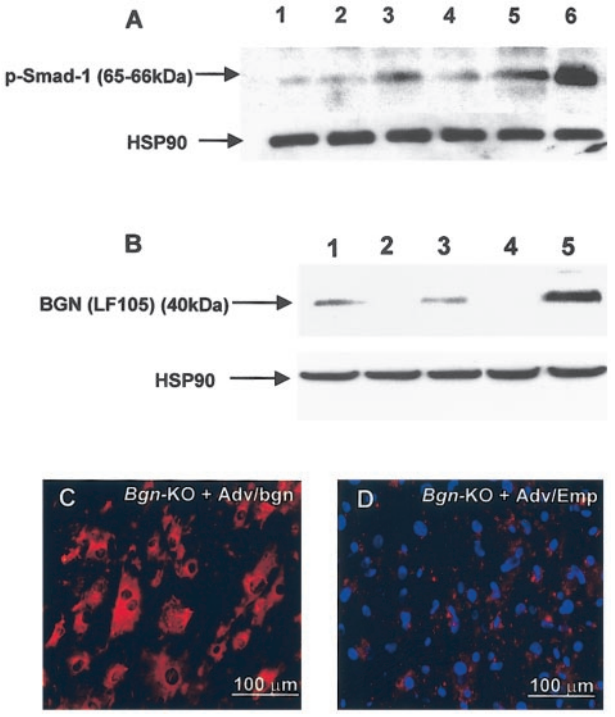

Figure 4. Bgn deficiency affected BMP-4 signal transduction. *A)* Western blot analysis of phosphorylated SMAD1 (p-SMAD1) in *WT* and *bgn*-KO neonatal murine calvarial cell cultures. A band of 65–66 kDa corresponding to p-SMAD1 was detected. Lanes 1 and 2: untreated *WT* and *bgn*-KO cells, respectively; lanes 3 and 4: *WT* and *bgn*-KO cells treated with BMP-4 for 30 min, respectively; lane 5: *bgn*-KO cells infected with adenovirus containing a bgn cDNA driven by a CMV promoter (adv-*bgn*) and treated with BMP-4 for 30 min; lane 6: positive control for p-SMAD1, which were lysates obtained from Cell Signaling Technology. Down-regulation of p-SMAD1 was observed in *bgn*-KO cells (lane 4). However, p-SMAD1 in the *bgn*-KO cells was elevated after infection with Adv-*bgn* (lane 5). HSP90 (bottom panel) demonstrates equal protein loading. *B)* The success of infection was confirmed by Western blot with antibody (LF105) to bgn shown in lane 5. Each lane represents the same samples shown in panel *A*. HSP90 (bottom panel) demonstrates equal protein loading. *C)* By immunohistochemical staining, *bgn*-KO cells infected with Adv-bgn produced bgn located in the cytoplasm. *D)* No bgn was detected in *bgn*-KO cell cultures, which were infected with an "empty" adenovirus that did not contain bgn gene (adv/Emp). Nuclear staining with DAPI is shown in blue.

that did not contain the *bgn* gene (*Bgn*-KO+Adv/Emp, Fig. 4*D*).

Cbfa1 transcriptional activation and differentiation is increased in *bgn*-KO cells infected with Adv-*bgn*

We measured the transcriptional activation of Cbfa1 in *bgn*-KO cells after infection with the Adv-*bgn* (**Fig. 5*A***). It was noticed that the addition of bgn elevated the basal line of luciferase activity in both *WT* and *bgn*-KO (WT/Adv-bgn and KO/Adv-bgn) compared with controls infected with the virus that did not contain *bgn* gene (WT/Adv-Emp, and KO/Adv-Emp). Furthermore, BMP-4 significantly increased the luciferase activity in the KO/Adv-*bgn*, but not in the KO/Adv-Emp. To determine whether the expression of osteoblastic markers could be increased in the *bgn*-KO cells (in addition to BSP and osteocalcin expression shown in Fig. 2), we assayed alkaline phosphatase (ALP) activity (Fig. 5*B*). When the *bgn*-KO cells were infected with Adv-*bgn* (KO/Adv-*bgn*), levels of ALP activity increased ~1.8-fold (from 20±3 to 35±4, $P<0.01$) by treatment with BMP-4. In contrast, the uninfected *bgn*-KO cells (KO/unit) increased only 1.3-fold (from 19±2 to 25±2, $P=0.047$) when treated with BMP-4. The *bgn*-KO cells infected with Adv-Emp (KO/Adv-Emp) did not

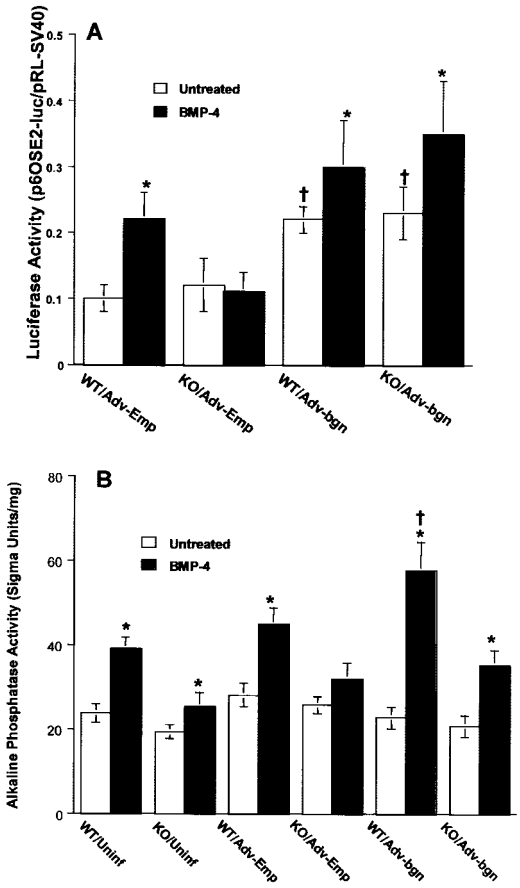

Figure 5. Cbfa1 transcriptional activation and differentiation in *bgn*-KO cells were rescued by infection with Adv-*bgn*. *A)* Cbfa1 transcriptional activation was rescued in *bgn*-KO cells after infection with Adv-*bgn* (KO/Adv-*bgn*) judged by a luciferase activity assay. As negative controls, *WT* and *bgn*-KO cells were infected with an "empty" adenovirus that did not contain a bgn gene (WT/Adv-Emp, KO/Adv-Emp). Levels of transcriptional activation were expressed as the ratio of luciferase activity of p6OSE2-luc/pRL-SV40. *$P < 0.05$ compared with the activity of cells with and without BMP-4. †$P < 0.05$ compared with cells between the infection of Adv-*bgn* and Adv-Emp (WT/Adv-*bgn* vs. WT/Adv-Emp or KO/Adv-*bgn* vs. KO/Adv-Emp) in the absence of BMP-4. *B)* Alkaline phosphatase activity was rescued in *bgn*-KO cells after infection with Adv-*bgn* (KO/Adv-*bgn*). WT/Uninf and KO/Uninf represent wild-type and *bgn*-KO cells without infection of Adv, respectively. *$P < 0.05$ compared with the activity of cells with and without BMP-4. †$P < 0.05$ compared with the activity of BMP-4-treated *WT* cells between those infected with Adv-*bgn* and Adv-Emp (WT/Adv-*bgn* vs. WT/Adv-Emp).

significantly increase ALP activity in response to BMP-4. This suggested that ALP activity in response of BMP-4 could be partially rescued in the KO cells by infection with Adv-*bgn*. BMP-4 increased ALP activity in *bgn* infected *WT* cells (WT/Adv-*bgn*) to a level that was greater than uninfected *WT* cells or *WT* cells infected with Adv-Emp.

Reduction of BMP-4 binding in the absence of bgn

To determine whether the partial block of BMP-4 signal transduction in *bgn*-KO cells was due to a change in expression of BMP receptors, we examined BMP type I receptors 1A and 1B by Western blot analysis; it is generally accepted that the type I receptor for BMP-2/4 plays a major role in high-affinity binding to its ligands even without coexpression of the type II receptor (30, 31). We found that *bgn*-KO cells overexpressed the receptor with or without BMP-4 treatment compared with *WT* cells. The down-regulation of receptor expression by BMP-4 was found in *WT* cells but not in KO cells (**Fig. 6*A***), suggesting that cells partially lost their ability to respond to BMP-4 in the absence of bgn. To address whether the overexpressed BMP type I receptor was due to an attempt by the cells to overcome a low affinity for BMP-4 binding, we examined BMP-positive cells using immunohistochemical staining. In this experiment, cells were incubated with BMP-4 for 2 h. After washing with PBS, we stained the cells using antibodies against BMP-2/4. The results showed that *WT* cells contained a greater number and intensity of BMP-4-positive cells compared with *bgn*-KO cells (Fig. 6*B*i vs. iii). Quantification of BMP-4-positive cells was performed by FACS analysis (Fig. 6*C*). Single-cell suspen-

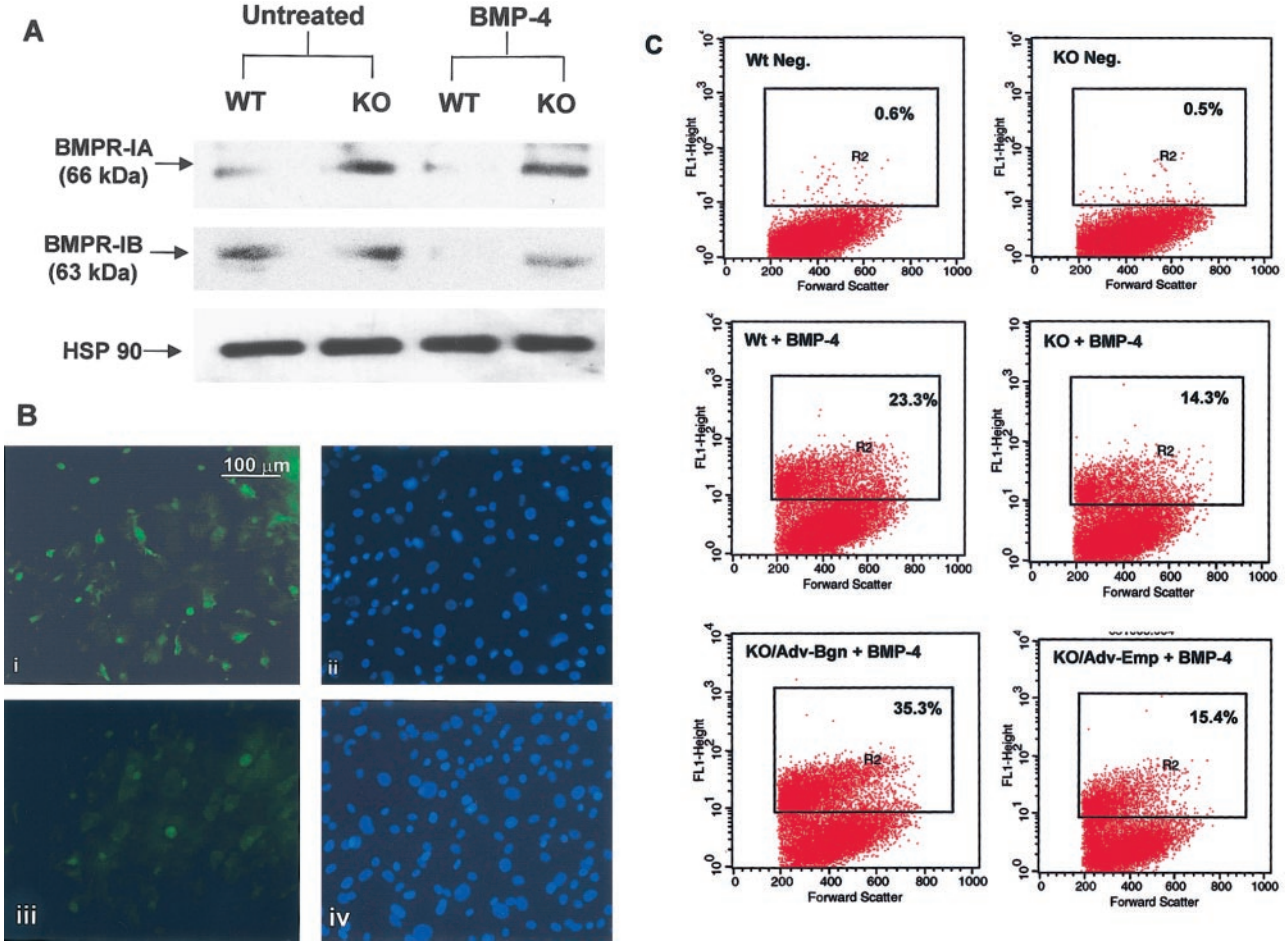

Figure 6. Bgn deficiency leads to a reduction in BMP-4 binding. *A)* Western blot analysis of BMP type I receptor (BMPR-1A and BMPR-1B) in *WT* and *bgn*-KO (KO) neonatal murine calvarial cell cultures in the absence or presence of BMP-4 for 24 h. HSP90 (bottom panel) demonstrates equal protein loading. *B)* Immunohistochemical staining to detect BMP-4-positive cells. The calvarial cells were cultured for 7 days, preincubated with BMP-4 for 2 h, and washed with PBS 3 times. i) BMP-4-positive cells with bright green fluorescence staining in *WT* cell cultures; ii) nuclear staining with DAPI shown in blue in *WT* cell cultures corresponding to the sample shown in picture i; iii) a significantly decreased number of BMP-4-positive cells with a dull green fluorescence staining in *bgn*-KO cell cultures compared with that in *WT* cell cultures are shown in pictures i; iv) Nuclear staining with DAPI in *bgn*-KO cell cultures corresponding to picture iii. *C)* FACS analysis to quantitate BMP-4 binding. Cells were grown to subconfluence, recovered by trypsin treatment, and preincubated with BMP-4. After washing, the cells were stained with antibody to BMP-4. Neg: Negative controls in which the cells were stained with antibody to BMP-4 without preincubated with BMP.

sions were prepared from monolayer cultures, preincubated with BMP-4, then washed three times in cold PBS. The BMP-positive cells were stained with antibody against BMP and analyzed by flow cytometry. The data showed that the *bgn*-deficient osteoblasts had fewer BMP-4-positive cells than *WT* cells (14% vs. 23%). However, when the KO cells were infected with Adv-*bgn*, the amount of BMP-4-positive cells increased to 35%. Endogenous BMP was determined by excluding the preincubation with BMP-4 and showed <1% BMP-positive reactivity in *WT* and *bgn*-KO cells.

Colocalization of bgn and BMP-4 binding, and decorin (dcn) in cultured calvarial cells

To further understand the relationship between bgn and BMP binding at a cellular level, we examined bgn expression in 7-day calvarial cell cultures using immunohistochemical staining. In *WT* cell cultures we found that bgn was localized in the cytoplasm, cell surface, and the matrix as shown by red fluorescence staining (**Fig. 7*A***). To find out whether BMP-4 binding paralleled the expression and localization of bgn, we stained cells with antibodies against BMP-4 and bgn using 2-color immunohistochemical staining. The colocalization of bgn (red) and BMP (green) were detected coincidentally, shown in the yellow or orange staining (Fig. 7*B*). This suggested that bgn could control BMP binding at the cellular level.

Since the structures of bgn and dcn are highly similar (12) and dcn may compensate for bgn's function in the *bgn*-KO, we theorized that the absence of bgn could change the distribution of dcn. We examined dcn in the cell cultures and found that the pattern of distribution for dcn in *bgn*-KO cell cultures was quite different from *WT* cell cultures. In *bgn*-KO cell cultures, dcn appeared to over-accumulate, and localized primarily in the matrix (Fig. 7*D*) compared with *WT* cell cultures (Fig. 7*C*).

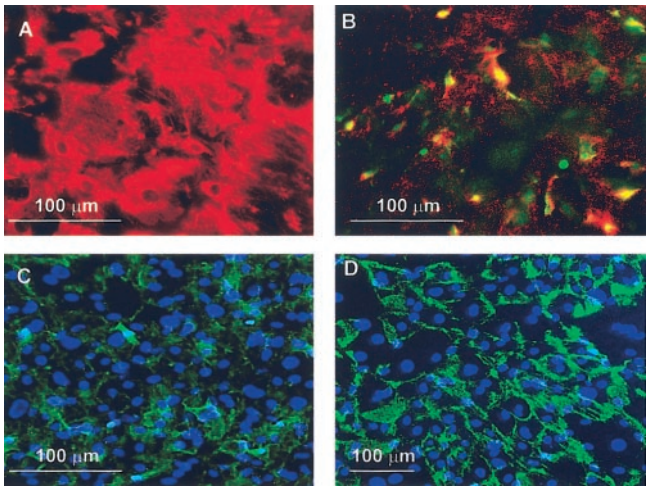

Figure 7. Distribution of bgn and dcn in neonatal murine calvarial cell cultures was determined by the immunohistochemical staining. *A)* In *WT* cell cultures, bgn (red) is localized in the cytoplasm, cell surface, and matrix. *B)* Colocalization of bgn (red) and BMP (green) were detected coincidentally as shown in yellow or orange staining. *C)* In *WT* cell cultures, dcn (green) is distributed close to the cells or in the matrix. Nuclear staining with DAPI is shown in blue. *D)* In *bgn*-KO cell cultures, dcn staining appeared to be denser, and mostly located in the matrix compared with that in *WT* cell cultures. Nuclear staining with DAPI is shown in blue.

DISCUSSION

Bgn-deficient mice develop age-dependant osteopenia resembling osteoporosis (15). The phenotype may arise from defects in the quantity and normal activity of bone marrow stromal stem cells (BMSSCs), which include increased apoptosis, causing a decreased number of BMSSCs and a poor response to TGF-β (16). Studies have suggested that TGF-β activity is modulated by bgn and its relative dcn. Based on the significant homology between TGF-β and BMPs, we speculated that *bgn* deficiency may also modulate BMP-induced osteogenesis. In this report, we demonstrate that bgn modulates the ability of BMP-4 to control osteoblast differentiation.

The absence of bgn impairs BMP-4-stimulated osteoblast differentiation

Bgn-KO cells produced less osteopontin than did *WT* cells and did not express BSP and osteocalcin, markers expressed in mature osteoblasts. In the presence of BMP-4, the *bgn*-KO cells accumulated less Ca^{2+} than *WT* cells. The basis for these phenotypes could be from an altered proportion of cell types found in the *WT* and *bgn*-KO cell culture or that *bgn*-KO cells lost their sensitivity to growth factors. To clarify this, it was necessary to indicate whether the above abnormalities could be rescued. The data showed that the *bgn*-KO cells could be completely rescued for expression of BSP and osteocalcin, and for their responsiveness to BMP-4. These results clearly suggest that the osteoblastic differentiation pathway might be partially blocked by a lack of bgn, resulting in maintenance of a preosteoblastic phase in *bgn*-KO cells and incomplete maturation into osteoblasts.

Since Cbfa1 is implicated in the regulation of gene transcription of osteopontin, BSP, and osteocalcin (26–28), we examined Cbfa1 in *bgn*-KO cells at the protein and transcriptional activation levels. It was clear that Cbfa1 protein levels in *bgn*-KO cells were lower than those in *WT* cells. A luciferase assay that measured Cbfa1 transcriptional activation indicated that BMP-4 could not significantly elevate Cbfa1 transcription in the KO cells compared with *WT* cells. However, there was no significant difference in the transcriptional activation by Cbfa1 between *WT* and *bgn*-KO cells without BMP-4 treatment, unlike what was seen at the protein level. This discrepancy may be explained by the differential sensitivities in the assays. The luciferase assay tested Cbfa1 transcriptional activation for 16 h

after the transfection of p6OSE2-Luc, which may be too short a period to see the difference between *WT* and *bgn*-KO cells vs. Cbfa1 protein levels that were measured in cells after 7 days of culture. We noticed that the increase Cbfa1 transcriptional activation in response of BMP-4 was not consistent with Cbfa1 protein levels, also observed by other investigators (32). Their data showed that ascorbic acid increases DNA binding and transcriptional activity of Cbfa1 without having significant effects on the expression of the Cbfa1 protein. It is likely that the induction of Cbfa1 transcriptional activation occurred much earlier that the changes in mRNA and protein levels. Taken together, the data indicate that bgn is important in modulating Cbfa1 transcriptional activation and subsequent osteoblast differentiation.

Bgn is essential for BMP-4 signal transduction in the stimulation of osteoblast differentiation

We invested phosphorylated SMAD1 activity, which is associated with BMP-4 signal transduction and has been implicated to regulate Cbfa1 (33), and found that *bgn*-KO cells produced less phospho-SMAD1 than *WT* cells after BMP-4 treatment. To address whether bgn was directly involved in the cell response to BMP-4, we infected KO cells with Adv-*bgn*. Our results showed that the response of *bgn*-KO cells to BMP-4 was completely rescued at both the SMAD1 signal transduction level and its farther downstream consequence of enhancing Cbfa1 transcription. In addition, bgn expression rescued the KO cells in response to BMP-4 in the expression of the mature osteoblast markers ALP (Fig. 5), BSP, and osteocalcin (Fig. 2). Our also showed that *WT* cells infected with Adv-bgn dramatically increased levels of Cbfa1 transcription and ALP activity in response of BMP-4 compared with the uninfected *WT* cells. These results strongly suggest that bgn is required for BMP-4 stimulation and further confirm earlier observations that SMAD1 is associated with Cbfa1 regulation (34, 35).

Bgn supports BMP-4 binding

We theorized that bgn might be important for BMP binding. To test this, we examined the expression of the BMP type I receptors 1A and 1B for BMP-2 and 4 binding (36) and found that *bgn*-KO cells actually expressed higher levels of the receptor that could not be down-regulated by BMP-4 compared with *WT* cells. One explanation could be that the KO cells responded by overexpressing the BMP type I receptor in order to overcome a low affinity of BMP binding. It is tempting to speculate that the resistance to increased expression after BMP-4 treatment could be due to a loss of BMP-4 sensitivity in the absence of bgn. To test this theory, experiments must be performed that measure the binding affinity of BMP-4 with or without bgn using classic ligand receptor cross-linking and will be the focus of future work. As an alternative to this procedure, we incubated the cells with or without BMP-4 for 2 h. The positive cells were detected using antibodies against BMP-2/4 after rigorous rinsing with PBS. A greater number of brightly stained positive cells were detected in *WT* cell cultures than in the *bgn*-KO cultures. The quantitation of BMP-4-positive cells with FACS analysis further supported what we found using immunohistochemical staining. FACS analysis also indicated that the KO cells had increased BMP-4 binding after being rescued. No BMP-2/4 was found on the cells without preincubation with BMP-4. This suggested that the endogenously produced BMP was too low to be detected in this experiment. This observation provides a direct explanation of why *bgn*-KO cells respond poorly to BMP-4. That is to say, the decreased levels of BMP-4-positive cells in *bgn*-KO cultures was due to low-affinity BMP-4 binding, where BMP was easily washed away during staining.

To better understand how bgn supports BMP-4 binding at a cellular level, we examined the colocalization of bgn and BMP-4 binding in the cell cultures using immunohistochemical staining. It was found that BMP-4 binding was coincident with bgn, implicating that bgn might physically support BMP-4 binding to the cells. Bgn was found on the cell surface and in the matrix, similar to what was reported in vivo (14). The pericellular arrangement of bgn indicates that it may have regulatory functions at the cell surface. Although *bgn*-KO cells infected with Adv-*bgn* produced a relatively smaller amount of bgn compared with *WT* cells, it appeared on the cell surface and intra-cellular and was enough to rescue the BMP-4 intracellular responses in the *bgn*-KO cells. It is possible that bgn may play a different role in the different stages of cell maturation. In early stages, bgn may interact with growth factors in controlling cell activity. In later stages, it is conceivable that the majority of cells are undergoing apoptosis, leaving bgn behind, and allowing it to participate in the organization and microarchitecture of the matrix.

The absence of bgn may affect the cellular microenvironment by altering the distribution of other matrix proteins

There are several cysteine-rich (CR) protein domains that bind BMP-4 having anti-BMP activity (37). Because CRs are present in many ECM proteins, including type 1, II, III, and V collagens, the role of CRs could involve the modulation and regulation of growth factors. We propose that the absence of bgn might affect this kind of regulation by altering the distribution of similar matrix proteins. In *bgn*-deficient mice, collagen fibrils have a greater variability in size and shape (38). The present study shows that the pattern of distribution of dcn was quite different in the *WT* and *bgn*-KO cell cultures (Fig. 7*C, D*). Dcn accumulated to higher levels in *bgn*-KO cell cultures and localized unevenly in the matrix compared with the *WT* cell cultures. This finding demonstrates that the pattern of distribution of dcn is changed when bgn is absent. Thus, one consequence of missing a single protein could be amplified by changing the distribution of other matrix proteins in a

"domino effect." The cell behavior therefore would be affected by a subsequent change of the cell microenvironment.

A model for the role of bgn in modulating BMP-4-stimulated osteoblast differentiation

Based on these observations, we propose a model to explain how bgn may modulate BMP-4 in controlling osteoblast functions (**Fig. 8**). It has been reported that bgn and dcn can bind TGF-β and that dcn inhibits TGF-β activity (39). Since BMP-4 is a member of TGF-β superfamily, we speculate that the similar structural motifs in bgn and dcn may competitively bind BMPs and balance a supply of those growth factors in a complementary way. Our data showed that dcn was mostly localized in the matrix, distant from the cells, as compared with bgn. Thus, dcn may sequester the BMP preventing binding to BMP receptors when bgn is absent. The predicted outcome would be a reduction in sensitivity to BMP-4, which could affect BMPs' signal transduction, causing a reduction of Cbfa1. This in turn could ultimately impair osteoblastic differentiation. Although our study indicates that bgn is a potential BMP binding protein, additional experiments are needed to understand the intricacies of how small proteoglycans bind and regulate growth factor functions.

In this report, we have demonstrated that bgn is required for optimal BMP stimulation. This leads to the conclusion that small proglycans may not only stabilize collagen fibrils, but may also balance growth factors to control osteoblast differentiation and function. FJ

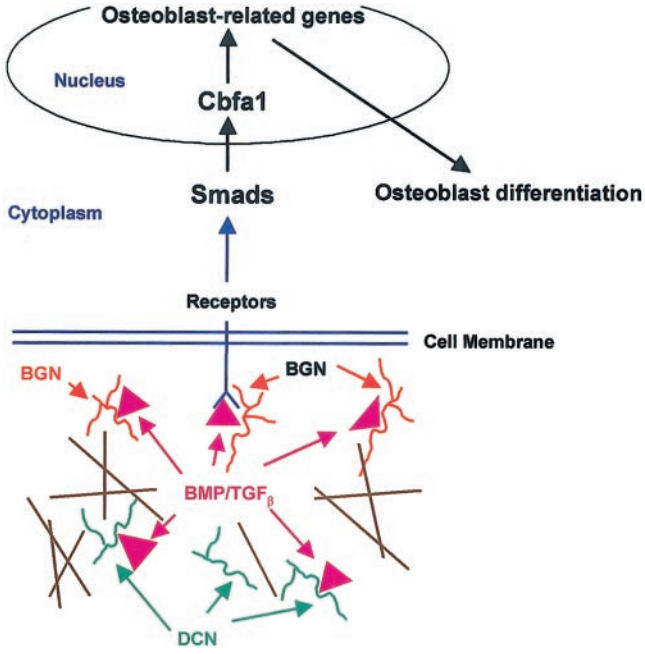

Figure 8. A proposed model of bgn and dcn modulation of BMP and TGF-β in controlling osteoblast functions. Bgn and dcn may competitively bind BMPs and TGF-β and balance the supply of those growth factors to the target cells in a complementary way. When bgn is absent, Dcn may sequester the BMPs away from cells because of their differential distributions. It would be a reduction in sensitivity to BMP-4, which could affect BMPs' signal transduction, thereby causing a reduction of Cbfa1. This, in turn, could ultimately impair osteoblastic differentiation.

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